Infrared Spectra and Protein Conformations in Aqueous Solutions

II. SURVEY OF GLOBULAR PROTEINS

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SUMMARY

Infrared spectra in the region of the amide I′ band have been determined for a number of proteins in D_2O solution. Comparison of the observed band positions with structural analyses based on optical rotatory dispersion and circular dichroism data, as well as with crystallographically determined structures, indicates that this technique may be useful in conformational studies of proteins in solution.

Conformational investigations of proteins and synthetic polypeptides in the solid state by means of infrared absorption spectroscopy have led to a considerable amount of valuable information (1–8). Similar studies on globular proteins in aqueous solution are greatly complicated by (*a*) the strong absorption of H_2O in the frequency range of interest, and (*b*) by the uncertainty of the magnitude of band shifts when, to avoid the first difficulty, H_2O is replaced by D_2O as solvent (8, 9).

The systematic investigation, described in the previous paper (10), of the manner in which the position and fine structure of the amide I band of three standard conformations (α-helix, antiparallel-chain pleated sheet, unordered) is affected by (*a*) dissolution in water, and (*b*) replacement of H_2O by D_2O, along with a consistent analysis of the observations in terms of the Miyazawa-Krimm (11–13) theory, has given some hope of making at least qualitative judgments about the conformations of globular proteins dissolved in D_2O. The infrared spectra of a number of proteins in D_2O solution have been obtained in the frequency range of 1500 to 1750 cm^{-1} and examined in terms of the band assignments made in the previous paper. The results are presented here, along with spectra of some less frequently encountered typical polypeptide conformations and the results of experiments carried out on proteins which had been caused to undergo conformational changes.

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EXPERIMENTAL PROCEDURE

The infrared spectra of protein solutions were obtained as described in the previous paper (10) at concentrations of 4 to 20 g per liter. The reported pD values were obtained by adding 0.40 (14) to values read on a Radiometer[1] model 4 pH meter. The β-lactoglobulins were materials prepared from the milk of typed homozygous cows (15); poly-L-proline was a sample kindly donated by Dr. W. F. Harrington. Other proteins and polypeptides were commercial preparations from the following sources: D_2O, BioRad, control No. 3135; γ-globulin, Sigma stock No. BG-11, Fraction II; crystallized bovine serum albumin, Mann, K1486A; carboxypeptidase A, Worthington; lysozyme, crystallized three times, Mann; recrystallized insulin, Mann; glucose oxidase, Calbiochem; DNAse, crystallized once, Worthington D1267-70; bovine carbonic anhydrase, Calbiochem; α-chymotrypsin, crystallized three times, Worthington; chymotrypsin, crystallized three times, Worthington; crystallized ribonuclease, Mann; soybean trypsin inhibitor, crystallized, Worthington; lyophilized rennin, Sigma; phosvitin, Nutritional Biochemicals. These preparations were further purified where necessary.

The deuterated methanol used for β-lactoglobulin denaturation studies in mixed D_2O, CH_3OD solution was purchased from New England Nuclear (lot No. 301-33-1). Stock solutions were adjusted to proper acidity by adding a small quantity of 12 N HCl. The hydrogen content of the solutions was thus held at a minimum. The composition of CH_3OD-D_2O solutions was calculated in percentage by volume. The reference cells contained the same solvent without protein.

RESULTS AND DISCUSSION

Polypeptides—Before proceeding to the examination of globular proteins in D_2O solution, it seemed desirable to examine the amide I and II bands of some less common polypeptide conformations, such as poly-L-proline I and II and the left-handed α-helix (special solvents must be used to produce these confor-

[1] Mention of the above product does not imply endorsement by the United States Department of Agriculture over others not mentioned.

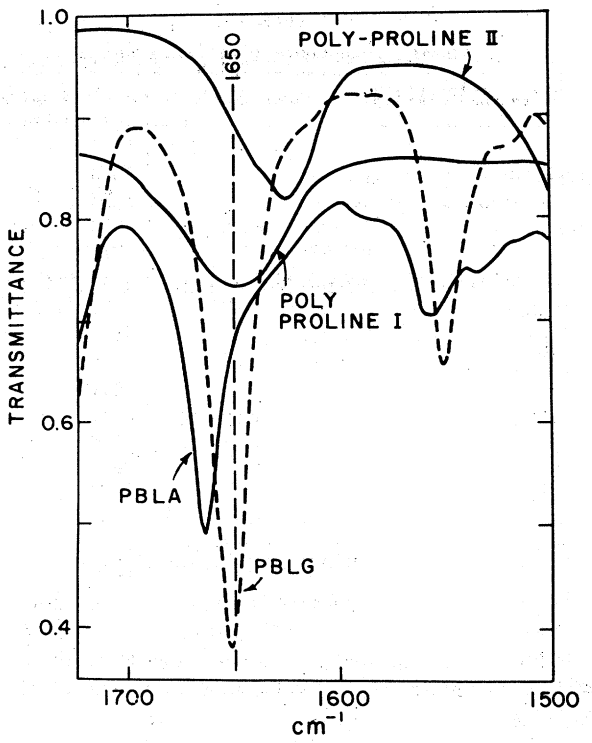


FIG. 1. Infrared spectra of some polypeptides. Polyproline I (in $CH_3(CH_2)_2OH$); polyproline II (in D_2O); poly-β-benzyl-L-aspartate (*PBLA*) (in $HCCl_3$); poly-γ-benzyl-L-glutamate (*PBLG*) (in $HCCl_3$).

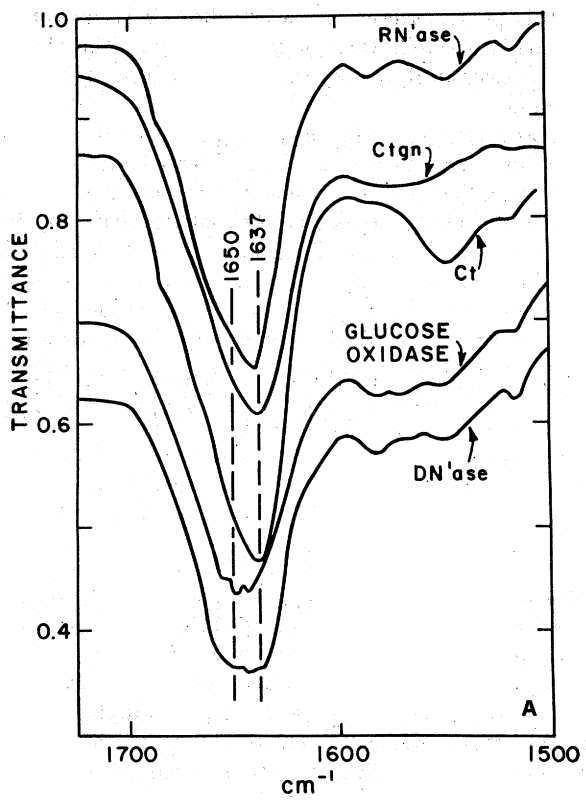
TABLE I

Amide I and amide II frequencies of some polypeptides

Polypeptide	Solvent	Amide I	Amide II
		cm^{-1}	cm^{-1}
Poly-β-benzyl-L-aspartate...	$HCCl_3$	1664	1557
Poly-γ-benzyl-L-glutamate...	$HCCl_3$	1651	1550
Polyproline I.....	$CH_3(CH_2)_2OH$	1649	
Polyproline II.....	D_2O	1625	

mations). These spectra are shown in Fig. 1 and the band positions are tabulated in Table I. The spectrum of poly-β-benzyl-L-aspartate in chloroform is of particular interest; in this medium, poly-β-benzyl-L-aspartate is known to be in a left-handed α-helical conformation (16, 17). The amide I band centers at 1664 cm^{-1}, *i.e.* it exhibits a high frequency shift of 13 wave numbers from the position of the corresponding band of right-handed α-helical poly-γ-benzyl-L-glutamate, which in the same solvent centers at 1651 cm^{-1}. This shift to a higher frequency appears to reflect the lower stability of the left-handed α-helix (18, 19) and is in agreement with recent results of Miyazawa *et al.* (20).

The infrared spectra of the two helical forms of poly-L-proline (Forms I and II) are characterized by amide I bands at 1649 and 1625 cm^{-1}, respectively, when these are examined in 1-propanol (Form I) and D_2O (Form II). The difference in the band positions may reflect differences in intrinsic structural characteristics as well as interaction with the two solvents. In polyproline, there are no C═O----HN hydrogen bonds; therefore, hydrogen bonding of the carboxyl groups to solvent does not necessarily



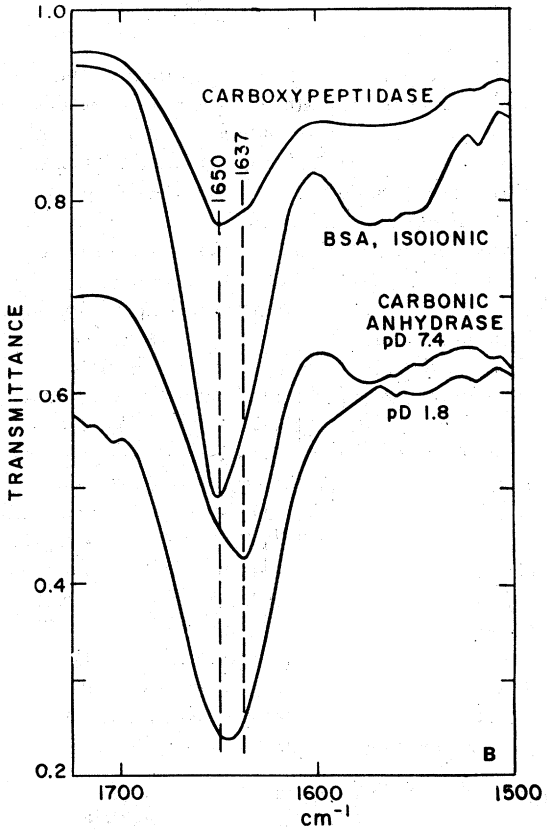


FIG. 2. Infrared spectra of various proteins in D_2O solution. *Ctgn*, chymotrypsinogen; *Ct*, α-chymotrypsin; *BSA*, bovine serum albumin; *RN'ase*, ribonuclease; *DN'ase*, deoxyribonuclease.

disrupt the polypeptide conformation, although it may contribute to a general destabilization of the structure. The absence of amide II bands in these spectra reflects the lack of NH groups in poly-L-proline.

Various Proteins—Typical spectra obtained with a number of proteins are shown in Figs. 2 and 3; observed amide I′ frequencies are listed in Table II. Cursory examination of these data shows that globular proteins in D_2O solution have highly distinct spectra in the amide I region, each protein exhibiting a band with a characteristic shape. In most cases, the band has a sharp maximum, with skewness exhibited at one side or the other. Much reproducible detail is frequently observed. For example, in glucose oxidase and deoxyribonuclease (Fig. 2*A*), the band is broad and displays several marginally resolved maxima of essentially equal intensity. Since these details are reproducible, they must be manifestations of real structural features. In general, it appears to be possible to divide these spectra into distinct classes. The great majority fall into one of two categories (*a*) those with maxima close to 1650 cm^{-1} and (*b*) those with maxima close to 1637 cm^{-1}. The first position is characteristic for an α-helical conformation (10–13), and encompasses proteins such as myoglobin (10, 24), lysozyme (21), and bovine serum albumin (Fig. 2*B*), in which this conformation is known to be present in considerable amounts. The second class consists of a large number of proteins which, within the limits of the present interpretation of optical rotatory dispersion data, have a structure that is not known to be dominated by any particular ordered conformation. It would seem, therefore, of interest to compare the infrared data of this class of proteins with corresponding data obtained by optical rotatory dispersion and circular dichroism. It does seem worthwhile to note, first, that polypeptides and proteins in the antiparallel-chain pleated sheet conformation (β structure) exhibit a strong amide I component around 1630 cm^{-1} and a much weaker one close to 1680 cm^{-1} (9, 10, 12, 13). The observed absorption maximum around 1637 cm^{-1} could be the result of overlapping, unresolved absorption bands arising from different conformations (such as an antiparallel-chain pleated sheet band superimposed on one due to unordered conformation). Thus, the frequency does not necessarily represent a band center characteristic of a specific conformation, a situation analogous to that found in other spectroscopic techniques, such as ultraviolet circular dichroism and optical rotatory dispersion.

Optical rotatory dispersion spectra have been obtained for a number of proteins and recently many of these have been summarized and classified (25–27) in terms of an apparent content of right-handed α-helix. If the infrared data of Table II are

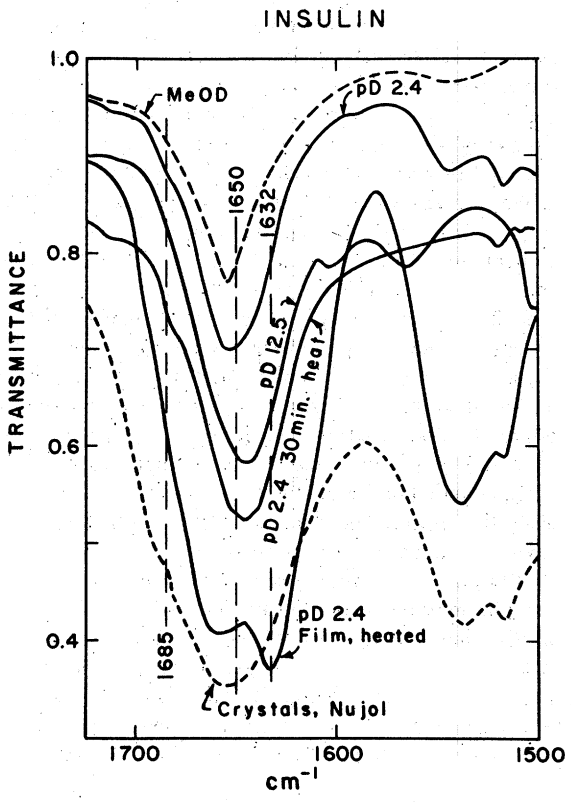


FIG. 3. Infrared spectra of insulin under various conditions

TABLE II

Amide I frequencies of various proteins

Protein	Conditions[a]	Frequency
		cm^{-1}
Myoglobin	pD 7.4	1650
Bovine serum albumin	Isoionic	1652
	Nujol suspension	1652
	pD 2.0	1648
Carboxypeptidase A	2.4 M LiCl	1650, 1637 shoulder
Lysozyme	Unspecified (21)	1650, 1632 shoulder
Insulin	pD 2.4	1654
	Nujol suspension	1654
	pD 12.5	1644
	pD 2.4 film, heated	1633, 1658
	Acidic CH_3OD	1654
Glucose oxidase		1643, 1648, 1654
Deoxyribonuclease		1637, 1643, 1650
Bovine carbonic anhydrase	pD 7.4, 12.0	1637
	Nujol suspension	1637
	pD 1.8	1646
α-Chymotrypsin	pD 4.2	1637, 1685 shoulder
Chymotrypsinogen	pD 8.7	1637
Ribonuclease	pD 4.8	1640, 1685 shoulder
Soybean trypsin inhibitor	pD 7.5 to 10.5	1641
γ-Globulin	Oriented film (22) (unpolarized light)	1650, 1635 shoulder
	pD 1.6	1637–1643 (broad)
	pD 10.0 to 12.0	1637
Rennin	pD 1.8, 10.8	1639
β-Lactoglobulin A	pD 1.5–8.0,[b]	1632, 1675
	Nujol suspension	1632, 1690 shoulder
	pD 12.4	1643
	pH 12.3 (in H_2O)	1656
	90% MeOD	1649, 1620 shoulder[c]
β-Lactoglobulin B tryptic core		1615, 1643
α_s-Casein C	pD 9–11 (10)	1643
	pH 9 (in H_2O)	1656
Phosvitin	pD 3.4; 6.6 (23)	1650

[a] Dilute D_2O solution, unless specified otherwise.

[b] Genetic variants B and C give spectra identical with A.

[c] The shoulder is observed at 4 g per liter of protein; it is absent at 0.5 g per liter.

compared with the ORD[2] parameters of the same proteins, it becomes evident that the proteins with amide I maxima near 1637 cm^{-1} also have Moffitt-Yang b_0 constants (28) close to zero. The ORD spectra of these proteins in the wave length region below 240 mμ are of a rather complex shape, but show low values of rotation, almost one order of magnitude less than those encountered in highly helical materials. Characteristically, many of these proteins display a shallow negative trough in the 225 to 235 mμ region at which an α-helix possesses a deep trough. It is known (29, 30) that in aqueous solution poly-L-lysine in the antiparallel-chain pleated sheet conformation has a shallow trough at 232 mμ, while polypeptide films in the same conformation have weak minima in the same wave length region (29, 31). Furthermore, the intrinsic b_0 parameter for an antiparallel-chain pleated sheet structure is known to be close to zero (32–36). The position of the amide I′ band close to 1637 cm^{-1} for this group of proteins appears to be consistent with optical rotatory dispersion data, if viewed within the framework of a significant contribution from a β structure, probably highly deformed and strained. The shift of the band toward higher frequency (1637 cm^{-1}) than that assigned (1632 cm^{-1}) (11–13) to a β conformation is consistent with a distortion of the structure due to steric hindrance and strains as well as superposition of the antiparallel-chain pleated sheet band on absorption stemming from unordered regions at about 1645 cm^{-1}. Furthermore, the stability of the pleated sheet conformation is thought to be closely related to side chain interactions (30).

An analysis of protein structure based on the amide I band is subject to the same limitations as those found in other spectroscopic techniques, such as ORD and CD, namely poor band resolution, overlapping of bands resulting in peak displacements, sensitivity of the exact band position to small variations of the different structures, and degree of interaction with solvent. The present infrared results frequently cannot distinguish between parallel and antiparallel pleated sheets because the characteristic antiparallel-chain pleated sheet band around 1680 cm^{-1} (10, 13) is not always easily observed in solution. Pysh (37) has calculated, however, the expected positions of the circular dichroic ultraviolet bands for these two conformations with the conclusion that these positions differ sufficiently to permit a distinction to be made between the two structures. Fasman and Potter (38) have carried out a systematic ORD examination of films of a number of polypeptides which are known to assume a β conformation; they report a rather complicated pattern of maxima and minima,[3] somewhat reminiscent of the spectra observed with a number of proteins (26). Except for cases of a clear-cut predominance of a single type of conformation, it is quite hazardous to draw definite conclusions on the conformation of a protein from infrared or ORD data alone. It does appear, however, that systematic application of the two techniques may lead to at least qualitatively correct interpretations.

A few detailed remarks on some of the proteins listed in Table II seem to be in order. The three-dimensional structures of ribonuclease and α-chymotrypsin stemming from x-ray diffraction analysis have been reported recently (39–42). These structures contain little α-helix; in accord with this fact is the absence of a distinct band at 1650 cm^{-1} (Fig. 2*A*), although a shoulder is clearly discernible in both proteins. In the crystal structure of these proteins there seem to be regions in which segments of the chains run in an essentially antiparallel fashion (a distorted β conformation) relative to each other; it appears to be relevant that a shoulder at 1680 cm^{-1} is clearly evident in the infrared spectra of both proteins. Furthermore, both the ORD (43–45) and CD[4] spectra of these proteins are consistent with the presence of little α-helix, the structures being composed primarily of unordered and β conformation. The absence of major α-helical regions from the structure of chymotrypsinogen (46) is borne out by the position of the amide I absorption maximum at 1637 cm^{-1} (Fig. 2*A*), which is consistent with the ORD and CD analyses of this protein (44, 45). In the case of carboxypeptidase A, the x-ray analysis suggests α-helical contents of about 30% (47), similar to the value found in lysozyme (48); the shape of the amide I band (Fig. 2*B*) with a maximum at 1650 cm^{-1}, and a shoulder at 1637 cm^{-1} is also similar to that of lysozyme (21), as is the ORD curve (49–52).

In the case of bovine carbonic anhydrase (Fig. 2*B*), the amide I band is found at 1637 cm^{-1} with a shoulder at 1650 cm^{-1}, in neutral or alkaline pH, as well as in a Nujol suspension of crystals; it shifts to 1647 cm^{-1} in the acid region. These results may be compared with the reported ORD (53–57) and CD (55) spectra obtained with the human and bovine enzymes; the latter have led to the conclusion that, in acid medium, this enzyme undergoes a transformation from a native structure to one containing about 20% α-helix (55). The ORD spectra of native and alkali-denatured materials do not lend themselves readily to conformational analysis due to interfering strong bands stemming from side chain chromophore transitions. A striking feature of the infrared results on the bovine enzyme is the essential identity of the amide I spectra obtained with this protein at pD 7.4 and 12.0, pointing to a lack of major structural alterations when the pH is raised. The similarity of the native carbonic anhydrase infrared spectrum to those of ribonuclease and chymotrypsin suggests the presence of some β structure. Such a situation appears consistent with the ORD spectrum of the native protein when the latter is compared to the ORD spectra of β-structured polypeptides (29).

Insulin in the native state has an amide I band maximum at 1654 cm^{-1} (Fig. 3 and Table II). This position is higher than those exhibited by the right-handed α-helical, antiparallel-chain pleated sheet, and unordered conformations within proteins in a D_2O medium. Lindley and Rollett (58) have suggested that insulin may contain α-helices of both senses (59). The amide I band position of poly-β-benzyl-L-aspartate (left-handed helix) is 13 cm^{-1} above that of poly-γ-benzyl-L-glutamate (right-handed helix) (Table I; see also Miyazawa *et al.* (20)), rendering the insulin spectrum consistent with this structural hyothesis. This hypothesis is also consistent with the ORD (26, 60, 61) and CD (62–64) data for this protein, although the latter results may be accounted for, as well, by a proper combination of α-helical, antiparallel-chain pleated sheet, and unordered structural regions. Denaturation of insulin in acidic methanol results in no change in the shape or position of its amide I band (Table II), a situation identical with those found in ORD (26, 61) and CD.[4] Exposure to pD 12.5 shifts this

[2] The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism.

[3] These observations have been confirmed recently by CD experiments, G. D. Fasman, L. Stevens, R. Townend, and S. N. Timasheff, manuscript in preparation.

[4] S. N. Timasheff and L. Stevens, manuscript in preparation.

band to 1644 cm^{-1} (Fig. 3), as expected for a solvent-penetrable unordered protein (10, 24). When insulin is heated at acid conditions, it forms fibrils (65) which are in a cross-β (cross-antiparallel-chain pleated sheet) structure (66). The spectrum obtained after 30 min of heating at 60° at pD 2.4 is shown in Fig. 3. The amide I band has become broadened with a simultaneous shift toward lower frequencies (apex at 1645 cm^{-1}) with a weak shoulder at 1680 cm^{-1}. Further heating resulted in precipitation. A suspension of the precipitate was cast as a film, the spectrum of which is also shown in Fig. 3. The resulting, amide I band is a doublet, with maxima at 1632 and 1658 cm^{-1} These spectra can be interpreted in terms of partial conversion, the film being composed of approximately equal amounts of an antiparallel-chain pleated sheet structure and some other conformation, or conformations. It is interesting that under similar conditions the ultraviolet circular dichroism spectrum shifts from one with a positive maximum at 192 mμ to one with a bimodal band with apices at 191 and 198 mμ (63), consistent with a transition from an α-helical to an antiparallel-chain pleated sheet structure. Thus, insulin may serve as an example of shifts in the amide I band which occur during different types of denaturation.

Native β-lactoglobulin has been shown (10, 24) to have a spectrum indicating an appreciable amount of antiparallel-chain pleated sheet structure. Furthermore, all known variants of this protein have essentially identical infrared spectra. In the present study, its alkaline denaturation in H_2O and D_2O and denaturation in a structure-inducing solvent, namely acidic deuterated methanol (CH_3OD), have been examined systematically.

The amide I spectra in D_2O with progressively increasing pD are shown in Fig. 4*A*. Above pH 9.7, irreversible structural changes occur (67) and the amide I band shifts gradually from the position of the native protein to higher frequencies. At pH 12.4, this protein is almost completely denatured, although complete exposure of tyrosine residues does not occur until pH 13 (68), and the amide I band has shifted to 1642 cm^{-1} or to the same position as obtained with α_s-casein (10), which is regarded as a disordered structure (69). The corresponding band shift with pH in H_2O is shown in Fig. 4*B*. The curves were obtained in a 0.01-mm cell using ordinate scale expansion (*cf.* "Experimental Procedure" of the preceding paper (10)). Thus, the gradual disordering of a protein structure can be followed by shifts in the amide I band in D_2O as well as in H_2O solution, although the entire shift is only about 10 cm^{-1} in the former and 20 cm^{-1} in the latter; furthermore, the shift of the band position shown in Fig. 4*A* parallels well the change in the optical rotatory parameter, a_0, over the same pH range (70).

A gradual change in medium from aqueous to methanolic transforms β-lactoglobulin from its native structure to one which is predominantly α-helical (70). The effect of this transition on the position of the amide I band was followed by gradually increasing the solution content of acid CH_3OD. Some spectra obtained at a protein concentration of 4 g per liter are shown in Fig. 5. At 30% CH_3OD, the structure is essentially that of the native protein. Above that solvent composition, a sharp change takes place both in the shape and position of the band. At 40% CH_3OD, the maximum absorption is at 1644 cm^{-1}, gradually increasing to 1649 cm^{-1} in 90% CH_3OD, *i.e.* the spectrum shifts toward predominance of α-helix. In the spectra obtained at 40% CH_3OD and above, a prominent shoulder is

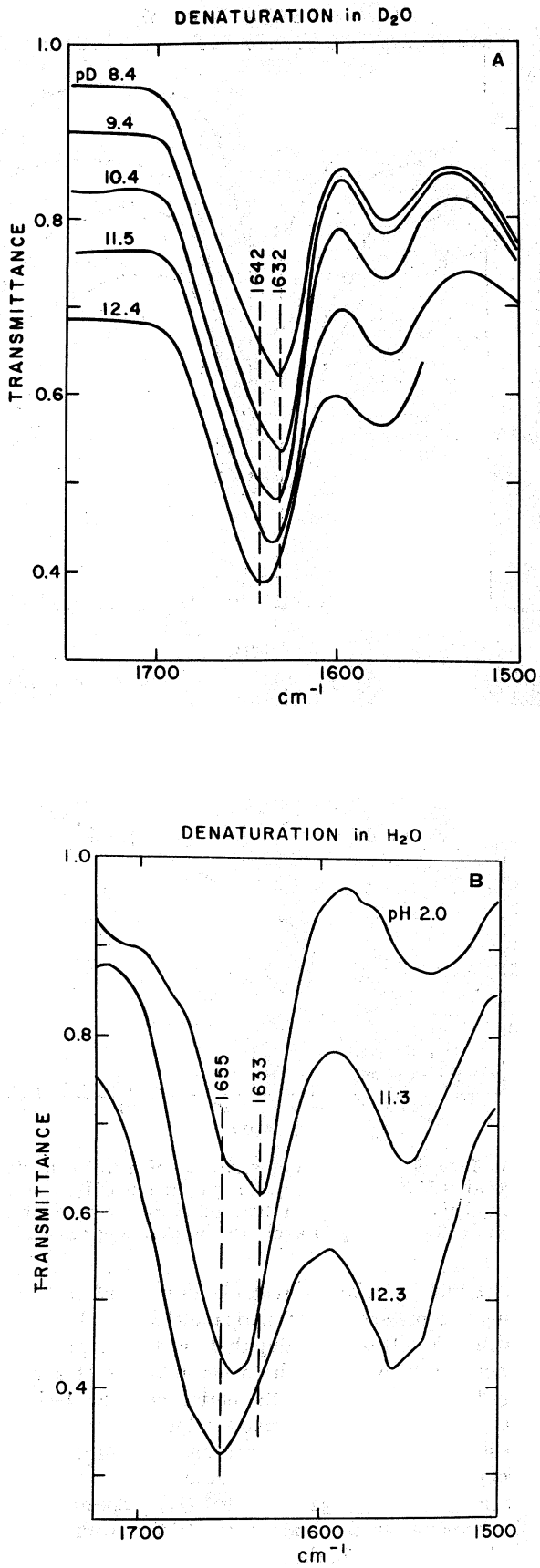


FIG. 4. Amide I band shift during alkaline denaturation of β-lactoglobulin A in D_2O (*upper*) and in H_2O (*lower*).

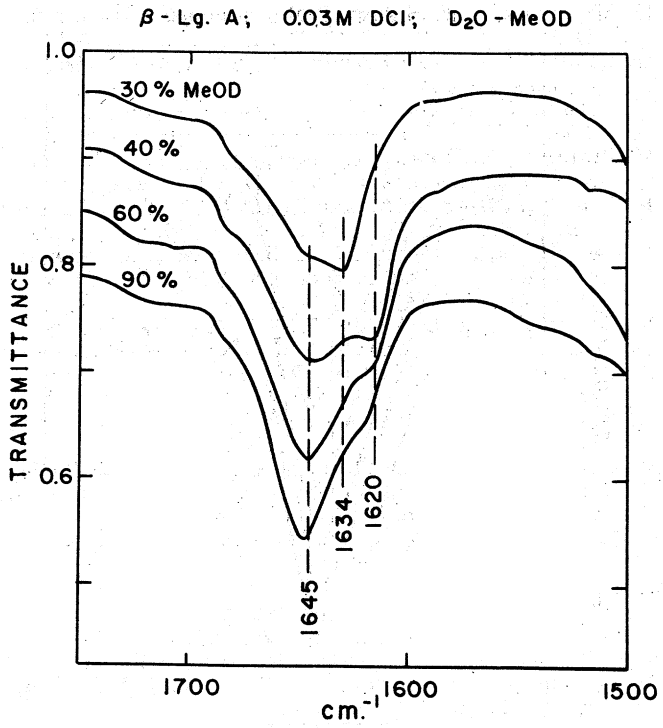


FIG. 5. Denaturation of β-lactoglobulin A (*β-Lg. A*) by adding CH_3OD to D_2O in the presence of 0.03 M DCl.

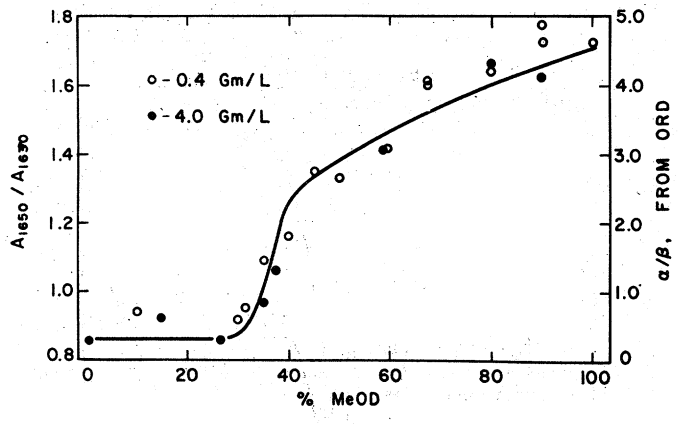


FIG. 6. Relative change of absorbance of β-lactoglobulin A solutions at 1650 and 1630 cm^{-1}, as a function of CH_3OD concentration. (The *points* at high protein concentration were obtained immediately after mixing to avoid the onset of aggregation.)

evident at 1620 cm^{-1}, and a weak one in the 1685 cm^{-1} region, indicating the presence of an antiparallel-chain pleated sheet (or β) structure. It is interesting that the new β structure band which appears upon methanolic denaturation is at a position different from that found in the native protein. Light scattering experiments (71) in methanolic solutions have shown that above 40% methanol, a 4 g per liter solution of β-lactoglobulin A undergoes a strong time-dependent aggregation. At 0.5 g per liter of protein, no aggregation occurs (71). Spectra were obtained, therefore, at the lower concentration with the result that no band appeared in the 1620 cm^{-1} region. Therefore, the band at 1620 cm^{-1} can be related to an aggregated species and probably corresponds to intermolecular β structure formation. This band is at a lower frequency than that of the intramolecular antiparallel-chain pleated sheet structure in the native protein, and is close to the corresponding band of synthetic polypeptides in contact with solvent (10). The described transition must be reflected in changes of the absorbance values at the characteristic frequencies. Since the specific amide absorbances of α-helical and antiparallel-chain pleated sheet structures are not known, the ratio of the absorbances at 1650 and 1630 cm^{-1} was followed as a function of CH_3OD concentration. The results are shown in Fig. 6 as $A_{1650}:A_{1630}$. The dependence of this ratio on methanol content was compared to the ratio of α-helical to β structure, deduced from optical rotatory dispersion measurements (72) which is shown by the *solid line* in Fig. 6. It is quite evident that the infrared data follow closely the ORD results, showing consistency between the two types of experiments on a semiquantitative basis.

CONCLUSIONS

The results presented in this and the preceding (10) paper suggest that infrared spectroscopy in the amide I band region can be helpful in the characterization in a general way of the structure of proteins in aqueous (D_2O) solution. In particular, it would appear that this technique can be used to best advantage to follow changes in conformation which result from changes in the medium, especially when used in conjunction with other methods, such as circular dichroism. Results obtained by a single technique are frequently difficult to interpret; the simultaneous application of several methods can often resolve such difficulties. It is evident that such solution techniques cannot yield as detailed results as x-ray diffraction measurements on protein crystals; nevertheless, the solution methods are of value in answering questions on the effect of placing the protein in dilute solution and on the kinds of conformational changes which occur when the solution medium is varied. At present, at best semiquantitative answers can be obtained to these questions. It is hoped that the availablility of more data on compounds of known structure will permit us to reach more detailed qualitative conclusions and, in particular, to identify the many features of fine structure evident in the spectra of a number of proteins.

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